ELSEVIER

Meat Science 54 (2000) 135–145

MEAT SCIENCE

www.elsevier.com/locate/meatsci

Combined use of Pronase E and a fungal extract (*Penicillium aurantiogriseum*) to potentiate the sensory characteristics of dry fermented sausages

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Received 24 March 1999; received in revised form 8 June 1999; accepted 15 June 1999

Abstract

A combination of an extract of *Penicillium aurantiogriseum* and Pronase E was used in an attempt to enhance the sensory characteristics of dry fermented sausages, specifically, a salami-like one named "salchichón". The addition of Pronase E alone (600 units/kg) produced a rise in free amino acids and biogenic amines and also an increase in the ammonia content. Addition of the protease and fungal extract (100.87 mg protein/kg of mixture) brought about a decrease in the level of free amino acids and a larger increase in the ammonia content than the batch added with only Pronase E. There was also an increase in the amount of specific volatile compounds such as 2- and 3-methylbutanal, 2- and 3-methylpropanal and 2- and 3-methyl-1-butanol produced by the breakdown of amino acids in these fermented sausages. Values of pH, a_w and dry matter were not affected by addition of the protease or fungal extract. In contrast, addition of Pronase E produced a notable change in the textural characteristics, reducing the hardness, cohesiveness, gumminess, chewiness and cutting force. Sensory analysis showed that fermented sausages manufactured with Pronase E and *Penicillium aurantiogriseum* extract had better odour, flavour, texture and, as a consequence, better general acceptability.

Keywords: Dry fermented sausages; Proteases; Moulds; Ripening

1. Introduction

Most of the sensory properties of dry fermented sausages can be attributed to the breakdown products of their main components, namely proteins and lipids, or to compounds produced by the further degradation of these products, i.e amino acids and free fatty acids, during the ripening process. For this reason, several authors have attempted to accelerate the ripening process by adding proteases and lipases of different origins (Blom et al., 1996; Díaz, Fernández, García de Fernando, Hoz & Ordóñez, 1993, 1996, 1997; Fernández, Hoz, Díaz, Cambero, Ordóñez, 1995a,b; Melendo, Beltrán, Jaime, Sancho & Roncalés, 1996; Zalacain, Zapelena, Paz de Peña, Astiasarán & Bello, 1997a,b; Næs, Holck, Axelsson, Andersen & Blom, 1995; Zalacain, Zapelena, Astiasarán & Bello, 1995, 1996; Zapelena, Zalacain, Paz de Peña, Astiasarán & Bello, 1997a,b). However, these studies were of limited success since, although the addition of enzymes produced an increased breakdown of proteins and lipids, resulting in the accumulation of free amino acids and fatty acids, little success was achieved in transforming these into compounds to achieve significant improvements in flavour and aroma (Díaz et al. 1997).

On the other hand, since moulds are part of the natural flora of several traditional fermented sausages, some authors have used fungal starter cultures in such meat products (Nuñez, Rodríguez, Bermúdez, Córdoba & Asensio, 1996; Toledo, Selgas, Casas, Ordóñez & García, 1997; Trigueros, García, Casas, Ordóñez & Selgas, 1995) and obtained a slight improvement in the sensory properties. These authors attributed this

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0309-1740/99/$ - see front matter
PII: S0309-1740(99)00076-5

improvement to lipolytic and proteolytic phenomena. Since these moulds are only applied to the surface of the products their action is limited. It is, therefore, reasonable to assume that the addition of a fungal enzymic extract to the fermented sausage mixture during manufacture would increase the accessibility of the mould enzymes to the components of the mixture. These enzymes would not only include proteases and lipases but also such enzymes as deaminases, transaminases and dehydrogenases which could help breakdown amino acids and form compounds which could contribute to the characteristic flavour and aroma of the final product.

Therefore, taking this data into consideration, it was suggested that the proteolytic enzymes, which produce an accumulation of the precursors of the volatile compounds, plus mould extracts from strains isolated from such sausages, which are capable of breaking down these precursors, would increase the amount of aromatic and sapid compounds generated and, as a consequence, improve the sensory properties of the fermented sausages. In the present work, an enzyme extract of *Penicillium aurantiogriseum* isolated from Spanish fermented sausages was used (Toledo et al. 1997; Trigueros et al. 1995). In a previous work (Bruna, Fernández, Hierro, de la Hoz & Ordóñez, 1999) we attempted to promote the transformation of the free amino acids by adding an extract from *Mucor racemosus* forma *sphaerosporus* to generate aromatic and sapid compounds to develop the aroma of fermented sausages. Although a higher score for overall acceptability was obtained in batches with added Pronase E and the extract of *Mucor racemosus* forma *sphaerosporus*, the results were not as good as expected. The present work studies the effect of adding Pronase E and a fungal extract of *Penicillium aurantiogriseum* on the sensory characteristics of dry fermented sausages.

2. Material and methods

2.1. Preparation of the extracts of Penicillium aurantiogriseum

An extract of an atoxigenic strain of *Penicillium* isolated from Spanish fermented sausages (Toledo et al., 1997; Trigueros et al., 1995) and identified as *Penicillium aurantiogriseum* by the International Mycological Institute (Egham, UK) was used. It was grown (22°C and pH 5.5 for 15 days) in several Roux bottles, each containing 250 ml of Czapeck Dox liquid medium (Oxoid, Unipath Ltd., UK) to give a final volume of 5 l. From the mycelium obtained by centrifugation ($1000\times g$, 15 min), 4 g aliquots were taken and, after mixing with 10 ml of ground glass and 14.5 ml of phosphate buffer, 0.2 M pH 5.5 were processed by a cellular disrupter (Braun MSK, Melsugen, Germany) for 2 min. The final mixture was filtered through Whatman No. 4 filter paper and the treatment procedure was repeated for the remaining mycelia. The filtrates obtained were combined and the resulting mixture was passed through a 0.45 μm filter (Millipore Corporation, Bedford, MA, USA) connected to a vacuum pump to eliminate the spores present in the extract. The filtered extracts were collected and the protein content, estimated by Lowry's method (Lowry, Rosenbrough, Farr & Randall, 1951), was 1.97 mg protein/ml.

2.2. In vitro experiment

The amino acid mixture used was formulated according to the amino acids released by 600 units of Pronase E in fermented sausages (Díaz et al., 1993) i.e: (Gly/Asn/His/Arg/Thr/Ala/Pro/Tyr/Val/Met/Ile/Leu/Phe/Trp/Lys/Asp/Glu/Ser/Gln) in the proportions (19/19/105/41/55/69/90/10/58/27/35/66/35/62/110/56/106/18/19). This was prepared in a phosphate buffer at pH 5.5 and 6.5 and each solution was divided into two parts. To one of these, 20 ml of the fungal enzyme extract were added (1.97 mg protein/ml) and to the other, the control, the same volume of phosphate buffer. The resulting solution was a 1% amino acid solution (10 mg/ml). The mixtures were sterilised by filtering through 0.45 μm pore diameter filters. Solutions were incubated at 12 and 22°C for 26 days, and samples taken at 0, 5, 15 and 26 days.

2.3. Preparation of the fermented sausages

The mixture for salchichon-type dry fermented sausages was prepared using the following formula: (% w/w): pork (55), beef (13.6), pork fat (2.5), NaCl (2.5), $NaNO_3$ (0.0085), $NaNO_2$ (0.0065), sodium ascorbate (0.046), lactose (1), glucose (0.8), dextrin (1.8) and equal amounts of whole grain and ground black pepper (0.14). The ingredients were processed in a mincer equipped with an adjustable plate set at a hole diameter of 5 mm and were inoculated with a starter culture of *Lactobacillus plantarum* 4045 and *Staphylococcus carnosus*. The total mixture was divided into four parts which were used to prepare four separate batches of fermented sausages: batch C (control) consisted of the starter mixture alone, batch PE of the starter mixture plus 600 units of Pronase E/kg mixture, batch PE + P was the same as batch PE but with addition of *Penicillium aurantiogriseum* extract (100.87 mg of protein/kg of mixture) and batch P was the same as batch C to which *Penicillium aurantiogriseum* extract had been added (100.87 mg of protein/kg of mixture). To equalise the moisture content of the batches, phosphate buffer was added to batches C, PE and P. After mixing, the mixtures were introduced into synthetic sausage casings,

40 mm in diameter and left to ripen in an Ibercex ripening cabinet, model G-28 (A.S.L., San Fernando de Henares, Madrid, Spain). The sausages were fermented at 22°C and 90% relative humidity (RH) for 12 h. After this, temperature and RH were slowly reduced to 18°C and 80% respectively in 60 h. Finally, the sausages were dried at 12°C and 80% RH until the end of the ripening process (a total of 26 days). The results reported here are the mean values obtained with samples from three separate manufacturing processes, carried out with different ingredients but the same formulation and technology.

2.4. Microbial analysis

The total viable count was done in Plate Count Agar (PCA) (Oxoid) and the *Micrococcaceae* count in Manitol Salt Agar (MSA) (Oxoid), both incubated at 32°C for 2 days. Lactic acid bacteria were cultivated in MRS agar (Oxoid) at pH 5.6 in a double layer at 32°C for 2 days.

2.5. Chemical analysis

The pH was measured in a homogenate of the sample with distilled water (1:10, p/v) using a Crison Digit-501 pH meter (Crison Instruments LTD, Barcelona, Spain). Dry matter (DM) was determined by drying the sample at 110°C to constant weight. Water activity (a_w) was determined using a Decagon CX1 hygrometer (Decagon Devices Inc., Pullman, WA, USA) at 25°C.

The total fat content of the samples was determined by cold extraction in chloroform and methanol in the presence of antioxidant BHT as described by Hanson and Olley (1963) and was quantified gravimetrically. Free fatty acids were quantified using chromatoplate densitometry (0.25 mm) with G-60 silica gel (Merck KGaA, Darmstadt, Germany) as described by Fernández et al. (1995a).

Free amino acids and amines were analysed as described by Díaz et al. (1993) and Ordóñez, de Pablo, Pérez, de Castro, Asensio and Sanz (1991), respectively. After extraction, amino acids were derivised with phenylisothiocyanate (PITC) and the amines with dansyl chloride. The derivatives were analysed in a Beckman System Gold *Nouveau* chromatograph (Fullerton, CA) equipped with a Waters column (Milford, MA, USA) Spherisorb S5 ODS2 (25 cm×4.6 mm, 5 μm particle size) maintained at 35°C. Detection was at 254 nm in both cases.

The eluents used for the amino acid analysis were: A, 0.03 M sodium acetate pH 6.8 to which triethylamine was added at 0.05% (v/v) and B, acetonitrile/water 90/10 v/v. Elution began with a mobile phase of 1 ml/min and an isocratic period of 5.5 min. After this, B was increased to 6% in 5 min, then to 15.5% in 9.5 min, 35% in 7 min and 38.5% in 12 min. The flow was then increased to 1.5 ml/min in 5 min, whereas B rose to 99% in 10 min. These conditions were maintained for 5 min. Initial conditions were then restored in 5 min.

The eluents used for amine analysis were as follows: A, 0.001 M sodium acetate and tetrahydrofurane 95/5 (v/v) at pH 4.2 and B, acetonitrile/water 90/10 (v/v). Elution was performed at a constant flow of 1.5 ml/min with an initial gradient of B of 50% which was gradually increased to 90% after 25 min. These conditions were maintained for 2 min after which B was reduced to 50% in 5 min. Finally, these conditions were maintained for 6 min.

Organic acids were analysed as described by González de Llano, Rodriguez and Cuesta (1996). For the extraction, 10 g of sausage mixture were homogenised with 20 ml of 4.5 mM H_2SO_4 in an ice bath. The mixture was shaken for 1 h at 10°C. After filtering (Whatman No. 54) the samples were refrigerated until analysed by HPLC. This consisted of first filtering the samples through 0.45 μm filters followed by injection of 25 μl on a Beckman System Gold *Nouveau* chromatograph equipped with an Aminex HPX-87H ion exchange column (300×7.8 mm) protected by a cation H^+ Microguard cartridge (BioRad Laboratories, Richmond, CA, USA) maintained at 65°C. Detection was at 210 nm. As eluent 3 mM H_2SO_4 was used. Elution was performed isocratically at an initial flow of 0.7 ml/min for 25 min after which it was increased to 0.9 ml/min up to 40 min. The different organic acids were identified using external standards, which consists of comparing the samples with standard solutions (Sigma Chemical CO, St. Louis, MO, USA) analysed under the same conditions.

Ammonia levels were determined using the Boehringer kit for enzyme analysis (Boehringer Mannheim GmbH, Mannheim, Germany) following the manufacturers instructions for meat products.

Volatile compounds were analysed by GC–MS. Extraction was done using a Tekmar 3000 dynamic head space system (Cincinnati, OH, USA). This involved the introduction of 10 g of the mixture in a vial heated to 40°C for 30 min, using helium as a carrier gas at a flow rate of 35 ml/min. Volatile compounds were collected in a Tenax trap cooled to −100°C with liquid nitrogen and then heated to 225°C for 5 min to desorb the volatile compounds before introducing them into the fused silica gel chromatograph column Quadrex Corporation (Woodbridge, CT, USA) (50 m×0.25 mm) filled with a 0.25 μm thick layer of FFAP, installed in a Perkin–Elmer gas chromatograph, model 8420 (Buckinghamshire, UK). For the separation an initial isothermal period of 40°C, followed by a gradient of 5°C/min was used until a temperature of 180°C was reached; this was then maintained for 15 min. The compounds were identified by comparing their retention times and mass

spectra with standards analysed under the same conditions and by comparison of the Perkin–Elmer ITD-50 Ion Trap Detector (electronic impact 70 eV) results with those recorded in the NBS library (National Bureau of Standards).

2.6. Textural analysis

This was done as described by Bourne (1978) in a TA.XT 2i/25 texture analyser (Stable Micro Systems, Surrey, UK) equipped with a cylindrical probe P/25 to determine hardness, cohesiveness, adhesiveness, gumminess, chewiness and springiness and a reversible probe to determine the maximum cutting force and the cutting work. This involved cutting samples approximately 1.5 cm long and 2.5 cm wide which were compressed twice to 50% of their thickness. The following parameters were defined: hardness (H) = maximum strength required to achieve compression; area of the first compression ($A1$) = total energy required for the first compression; area of the second compression ($A2$) = total energy required for the second compression, adhesiveness = area under the abscissa after the first compression; springiness (S) = height the sample recovers between the first and second compression; cohesiveness (C) = $A2/A1$; gumminess (G) = $H \times C$; chewiness (Ch) = $S \times G$; maximum cutting strength = maximum height on the cutting graph and total cutting work = area under the cutting curve.

2.7. Sensory analysis

This was carried out at the end of ripening by a panel of 18 tasters. These were all members of the Departamento de Higiene y Tecnología de los Alimentos, who had been previously trained in the sensory assessment of meat products. A triangular test was carried out (ISO, 1981) and also the colour, texture, odour and taste were assessed using a non-structured hedonic scale in which samples were given scores of 1 (very poor) to 10 (excellent). The overall quality was calculated from the expression: general acceptability = (color × 0.1) + (texture × 0.25) + (odour × 0.15) + (flavour × 0.5). This expression was calculated taking into account the opinion of the 18 tasters who, in a study on commercial fermented sausages, had been asked to assess the relative importance of the different organoleptic characteristics. Similar expressions have also been used previously (Fernández et al. 1995b; Díaz et al. 1996, 1997).

2.8. Statistical analysis

ANOVA was used to detect significant differences between means. Comparison between batches was performed using the Student Newman–Keul's test ($p < 0.05$).

3. Results and discussion

3.1. In vitro experiment

In the in vitro experiment (Table 1) there were no significant differences between the total free amino acid contents of the controls incubated at 12 and 22°C and at pH 5.5 and 6.5, with values around 9 mg/ml being recorded at the end of incubation, which were similar to those recorded at the beginning of the experiment. For this reason, only values corresponding to one of the control batches are given in the table. However, the total free amino acid contents changed significantly over the incubation period in batches to which *Penicillium aurantiogriseum* extract had been added. The initial amino acid content was significantly higher ($p < 0.05$) in these batches than in controls, probably due to the contents of the remaining culture medium or to the enzyme extract. Initial values of this parameter (12.32 mg/ml) dropped to values about 10 mg/ml in 5 days except for the batch incubated at 22°C and pH 5.5 for which a lower value was recorded. This decline continued over the entire incubation period to reach final values of 6.3 and 8.75 mg/ml. The most pronounced drop in amino acid content was observed in the batch incubated at 22°C and pH 5.5 (6.3 mg/ml). Breakdown was greater in the batches incubated at 22°C than in those incubated at 12°C and the most favourable pH was 5.5.

In the analysis of the individual amino acids, Gly, Ala, Ile, Leu and Val underwent the greatest decline, especially at pH 5.5. In the batch incubated at 22°C and pH 5.5 a reduction in Asp and Asn was also observed. These results and the total free amino acid values suggest that the fungal extract facilitates amino acid breakdown and this is more pronounced at 22°C and pH 5.5, conditions similar to those found in dry fermented sausages.

3.2. Experiments on fermented sausages

Addition of Pronase E and/or fungal extract did not affect the bacteria normally present in fermented sausages. Initial levels of total viable and lactic acid bacteria counts of around 10^5 cfu/g on the first day rose sharply in the first 4 days to reach values between 10^8 and 10^9 cfu/g, with little further change to the end of ripening. These results indicate that lactic acid bacteria were the dominant flora during ripening. The bacteria which developed in MSA (*Micrococcaceae*) stabilised at about 5 days at approximately 10^6 cfu/g. Similar results have also been reported by many authors, e.g. Lücke (1984), Díaz et al. (1993, 1996) and Fernández et al. (1995a).

Use of Pronase E and/or fungal extract did not affect the pH, dry matter or water activity of the fermented

Table 1
Changes in free amino acids (mg/ml) in the in vitro experiment during incubation at 22 and 12°C and pH 5.5 and 6.5

	Day 0		Day 5					Day 15					Day 26				
	C	P	C	P				C[a]	P[b]				C	P			
				pH 5.5		pH 6.5			pH 5.5		pH 6.5			pH 5.5		pH 6.5	
Amino acid				12°C	22°C	12°C	22°C		12°C	22°C	12°C	22°C		12°C	22°C	12°C	22°C
Asp	0.52a	0.53a	0.53a	0.42b	0.33b	0.52a	0.51a	0.37a	0.38a	0.21b	0.46c	0.47c	0.39a	0.35a	0.09b	0.41a	0.4a
Glu	1.1a	1.45b	1.04a	1.31b	0.97a	0.97a	1.15a	0.87a	1,23b	0.95a	0.95a	1.03a	0.97a	1.08b	0.88c	0.90a,c	0.83c
Ser	0.16a	0.18a	0.15a	0.12a	0.15a	0.12a	0.16a	0.41a	0.15b	0.12b	0.11b	0.16b	0.26a	0.12b	0.07b	0.10b	0.15b
Asn	0.23a	0.34b	0.26a	0.2a	0.28a	0.21a	0.20a	0.27a	0.21a	0.09b	0.17b	0.23a	0.48a	0.20b	0.08c	0.18b	0.18b
Gly	0.19a	0.37b	0.21a	0.34b	0.2a	0.35b	0.34b	0.20a	0.15b	0.23a	0.33c	0.32c	0.12a	0.18a,b	0.16a,b	0.25b	0.29b
Gln	0.15a	0.24a	0.14a	0.23b	0.22b	0.24b	0.23b	0.07a	0.18b	0.11a	0.25c	0.20c	0.27a	0.15b	0.12b	0.22a	0.16a,b
His	1.1a	1.27a	0.98a	0.97a	1.10a	1.15a	1.12a	0.68a	0.86b	1.02c	1.00c	0.95c	0.89a	0.67c	0.73b,c	0.83a	0.76b
Thr	0.54a	0.56a	0.54a	0.57a	0.49a	0.59a	0.57a	0.51a	0.55a	0.38b	0.55a	0.46a	0.47a	0.53c	0.37b	0.52c	0.44a
Ala	0.66a	0.66a	0.54a	0.39b	0.51a	0.40b	0.38b	0.49a	0.21b	0.45a	0.32b	0.32b	0.37a	0.11c	0.48b	0.33a	0.29a
Pro	1.1a	1.2a	1.23a	1.15a	1.08a	1.19a	1.13a	1.42a	1.06b	1.1b	1.15b	0.95b	1.21a	1.02b,c	0.98b,c	1.1b	0.85
Arg	0.45a	0.78b	0.41a	0.89b	0.56c	0.66c	0.62c	0.32a	0.86b	0.56c	0.68c	0.58c	0.07a	0.79b	0.47c	0.61d	0.63d
Tyr	0.09a	0.22b	0.10a,b	0.21a	0.04b	0.22a	0.21a	0.17a	0.21a	0.01b	0.15a	0.21a	0.22a	0.21b	0.00c	0.19b	0.21b
Val	0.62a	0.66a	0.66a	0.48b	0.17c	0.41b	0.39b	1.12a	0.51b	0.15c	0.39d	0.36d	0.37a	0.19b	0.16b	0.33a	0.3a
Met	0.26a	0.37b	0.17a	0.33b	0.31b	0.35b	0.34b	0.12a	0.2b	0.22b	0.31c	0.31c	0.08a	0.23b,c	0.13b	0.27c	0.23b,c
Ile	0.45a	0.57b	0.51a	0.37b	0.54a	0.53a	0.47a	0.45a	0.36b	0.39b	0.48a	0.44a	0.54a	0.19c	0.16c	0.51a	0.44a,b
Leu	0.78a	0.79a	0.74a	0.60b	0.34c	0.65a,b	0.6a,b	0.61a	0.49b	0.33c	0.58a	0.57a	0.87a	0.27b	0.26b	0.31b	0.5c
Phe	0.31a	0.45a	0.33a	0.37a	0.34a	0.46b	0.45	0.22a	0.33a,b	0.37b	0.41b	0.39b	0.57a	0.27b	0.16c	0.36b	0.27b
Trp	0.51a	0.58a	0.56a	0.51a,b	0.50a,b	0.66c	0.64c	0.5a	0.59a	0.51a	0.59a	0.59a	0.49a	0.51a	0.33b	0.51a	0.37b
Lys	0.98a	1.1a	0.87a	0.98a	0.75b	1.12a	1.10a	0.61a	0.92c	0.72b	0.87c	1.02d	0.86a	0.81a	0.67b	0.80a	0.67b
Total	10.2a	12.32b	9.97a	10.44b	0.88c	10.81a	10.64b	9.41a	9.45a	7.92b	9.75a	9.57a	9.5a	7.9b	6.3c	8.75d	7.97b

[a] C: Control solution. P: Control solution added with *Penicillium aurantiogriseum* extract (1.97 mg protein/ml).
[b] a, b, c: Values in a row with different letters are significantly different ($p < 0.05$).

sausages. The values and trends of all the parameters were similar to those described by Lücke (1984), Sanz, Selgas, Parejo and Ordóñez (1988), Nychas and Arkoudelos (1990), Fernández et al. (1995a), Díaz et al. (1996, 1997) and Bruna et al. (1999).

The fat content of the sausages (data not shown) was approximately 60% of the dry matter at the end of ripening, a value similar to that usually recorded in dry fermented sausages (Fernández et al., 1995a). The free fatty acid fraction followed a similar trend in all the batches, including controls (data not shown). Initial values of around 30 mg/100 g DM rose to between 150 and 170 mg/100g DM at the end of ripening. These results demonstrate that neither the fungal extract nor Pronase E were lipolytic. The levels of the free fatty acids were similar to those described by Toledo (1997), although they were lower than found in different fermented sausages by Demeyer, Hoozee and Mesdom (1974), Domínguez and Zumalacárregui (1991), Fernández et al. (1995a), León Crespo et al. (1985).

The total free amino acid content and the changes in each free amino acid during ripening are shown in Table 2. In the batches to which Pronase E had been added there was a sharp rise in the value of free amino acids during fermentation (day 5), from initial values of about 1000 mg/100 g DM to about 3800 mg/100 DM However, in batches not supplemented with Pronase E (control and batch P) this value was lower, at about 1500 mg/100 g DM at the end of the ripening. These values were significantly higher than those observed by other authors both in conventional fermented sausages and sausages manufactured using different proteases (Næs et al. 1995; Zapelena et al., 1997a). The amino acid content of the batches supplemented with Pronase E was slightly higher than recorded by Díaz et al. (1993, 1997), using a Pronase E treatment. At the end of ripening, the batches to which fungal extract had been added (P and PE + P) had significantly ($p < 0.05$) lower levels of total free amino acids (about 6–7% lower) than their corresponding controls (batches C and PE). This could be explained by a degradative effect of the fungal extract on the amino acids, which would accord with the results of the in vitro experiment (Table 1).

The amino acid profiles (Table 2) of the control fermented sausages and those manufactured with Pronase E were similar to those described by Díaz et al. (1993) for similar fermented sausages. Both types of dry fermented sausage (PE and PE + P) had a significantly higher content of Glu, Ala, Pro + Cys and Leu. There was an overall reduction of all the amino acids in the batches manufactured with fungal extract compared to batches manufactured without this extract. At the end

Table 2
Changes in free amino acids and ammonia (mg/100 g D.M.) during ripening of dry fermented sausages

	Day 0	Day 5				Day 15				Day 26			
Amino acid	C[a]	C[a]	PE[b]	PE + P[c]	P[d]	C[a]	PE[b]	PE + P[C]	P[d]	C[a]	PE[b]	PE + P[c]	P[d]
Asp	5.65	28.74a[e]	128.37b	126.66b	24.04a	73.06a	190.37b	182.53b	63.18a	71.31a	186.56b	203.04b	82.97a
Glu	29.11	146.35a	387.40b	358.11c	131.92d	356.30a	629.65b	673.99b	369.92a	345.63a	642.06b	637.76b	141.68c
Ser	12.88	28.93a	119.23b	106.93b	20.95a	55.52a	134.24b	111.54c	30.86d	78.35a	117.55b	101.49c	38.88d
Asn	5.81	6.84a	63.53b	66.52b	4.03a	17.61a	74.35b	73.96b	9.13c	23.69a	57.86b	99.93c	12.55d
Gly	35.02	58.69a	155.74b	145.05c	59.21a	82.68a	200.69b	205.79b	74.99a	80.45a	185.85b	148.09c	97.27d
Gln + Tau	423.53	210.88a	475.94b	457.57c	227.97d	83.95a	267.20b	209.67c	55.16d	50.62a	230.17b	213.84b	65.90a
His	12.83	33.45a	158.91b	161.98b	30.56a	30.93a	92.19b	102.70b	27.36a	81.06a	65.35b	91.14a	44.58c
Thr	8.23	39.67a	136.61b	134.41b	51.81c	41.54a	117.50b	135.84c	31.40d	66.90a	114.92b	119.29b	41.94c
Ala	70.11	118.96a	340.13b	343.29b	133.11c	163.59a	333.88b	313.24c	138.99d	218.51a	312.63b	289.28c	100.50d
Pro + Cys	342.68	421.78a	345.50b	364.50c	415.37a	402.46a	369.21b	366.88b	352.10b	416.08a	361.83b	346.09b	482.00c
Agr	9.88	12.09a	5.67b	6.53b	11.38a	3.16	15.28b	14.02b	4.56a	16.86a	15.54a	9.10c	3.92d
Tyr	9.29	14.13a	11.62b	11.40b	7.95c	18.92a	55.26b	44.36c	7.24d	16.56a	67.69b	34.48c	8.89d
Val	16.54	59.98a	241.48b	244.68b	69.17c	87.32a	249.20b	206.45c	76.56a	89.51a	244.22b	192.71c	77.52a
Met	0.95	25.04a	100.65b	102.20b	23.45a	40.49a	98.39b	107.85b	39.22a	53.78a	87.65b	99.36b	64.36a
Ile	11.64	35.01a	165.65b	153.70c	21.41d	58.23a	162.38b	180.61b	42.08a	52.36a	152.86b	163.88b	45.60a
Leu	23.92	92.77a	333.34b	341.54b	115.19c	124.72a	308.37b	310.39b	118.10b	134.14a	302.21b	263.22c	170.75c
Phe	11.38	47.74a	155.83b	145.47b	58.22a	59.35a	140.56b	110.91c	56.96a	88.02a	122.75b	117.02b	81.10a
Trp	5.42	49.00a	279.50b	239.95c	40.60a	58.66a	242.49b	214.62c	32.08d	79.08a	211.02b	214.08b	74.76a
Lys	33.08	52.15a	294.29b	265.18c	73.13a	101.91c	346.33b	318.34c	79.96b	179.62a	352.44b	288.55c	109.02a
Total	1067.97	1482.29a	3899.47b	3775.76c	1519.56a	1860.47a	4027.63b	3847.79c	1609.98d	2142.61a	3831.25b	3632.43c	2017.27a
NH_3	29.6	53.86a	63.20b	69.80c	54.60a	50.70a	62.50b	63.20b	52.00a	51.00a	67.90b	78.50c	66.30b

[a] C: Control batch.
[b] PE: Control batch added with 600 units/kg of Pronase E.
[c] PE + P: PE batch added with *Penicillium aurantiogriseum* extract (100.87 mg protein/kg, initial mixture).
[d] P: Control batch added with *Penicillium aurantiogriseum* extract (100.87 mg protein/kg, initial mixture).
[e] a, b, c, d: Values in a row with different letters are significantly different ($p < 0.05$).

of ripening, the amino acids which underwent the greatest reduction in batch PE + P in comparison to batch PE were Gly (20%), Arg (41%), Tyr (49%), Val (21%) and Lys (18%). However, in batch P, the amino acids most affected were Lys (39%), Tyr (46%), Arg (75%), Ala (54%), Thr (37%), His (45%), Asn (47%) and Ser (50%) compared to control batch C. In both cases (batches PE + P and P), the loss of amino acids was very marked, as percentages higher than 20% were transformed to other compounds by the action of the fungal extract. As no increases in amine content (PE + P vs PE and P vs C) were observed (Table 3, day 26), it may be deduced that the decreases found in the amino acid contents were due to transformation via deamination to yield the corresponding keto-acid, with the concomitant release of ammonia (Table 2). On the other hand, addition of the fungal extract appears to produce at day 26 a rise (not significant, $p > 0.05$) in the levels of Asp, Met and Gln + Tau in both cases and also a rise ($p < 0.05$) in Asn and His in batch PE + P and Glu, Gly and Leu in batch P ($p < 0.05$).

The profile of the ammonia content of the different batches of fermented sausages is also shown in Table 2. In all cases the ammonia content rose during the first 5 days of ripening, from initial values of 29.6 mg/100 g DM to 50–70 mg/100 g DM on the 5th day and then remained fairly constant to the end of ripening. Similar results have been reported by Dierick, Vanderkerhove and Demeyer (1974) and Huang and Lin (1992). The rise in ammonia content was recorded in both batches supplemented with Pronase E and the greatest rise was observed in the PE + P batch. The values recorded in the batches manufactured with fungal extract reflect changes occurring in the free amino acid content, corresponding to breakdown of the amino acids to produce ammonia and the corresponding keto-acid (Wellner & Lichtemberg, 1971). In the fermented sausages supplemented with Pronase E alone, the increased ammonia content was probably due to the increased metabolic activity of the microorganisms because of either the larger amount of available free amino acids or to residual aminooxidase activity of Pronase E.

Table 3 shows changes in the total amine content during ripening. The initial content of 21.3 mg/100 g DM rose slightly in batches C and P (to about 50 mg/100 g DM at the end of ripening) whereas the batches to which Pronase E had been added exhibited a sharp rise, even during fermentation reaching values of 122 mg/100 g DM in batch PE and 132 mg/100 g/DM in batch PE + P at the end of ripening. These increases indicate

Table 3
Changes in amines (mg/100 g DM) during ripening of dry fermented sausages

	Day 0	Day 5				Day 15				Day 26			
	C[a]	C[a]	PE[b]	PE + P[c]	P[d]	C[a]	PE[b]	PE + P[c]	P[d]	C[a]	PE[b]	PE + P[c]	P[d]
Triptamine	5.8	4.87a[e]	13.64b	14.25b	6.22a	6.07a	23.77b	24.0b	7.10a	4.92a	28.30b	30.20b	3.85a
Phenylethylamine	1.5	1.55a	4.16b	2.36a	2.72a,b	2.29a	5.38b	8.83c	2.01a	1.80a	6.23b	5.00b	2.24a
Putrescine	2.0	8.54a	24.64b	16.32c	10.88	19.39a	50.36b	76.80c	21.32a	21.98a	55.14b	64.00b	20.54a
Histamine + cadaverine	5.6	15.47a	27.13b	26.47b	13.60a	20.14a	24.54b	28.03c	27.60c	20.39a	28.70b	26.80b	21.71a
Tyramine	0.2	2.71a	3.50a	5.89b	0.68c	0.71a	3.22b	3.64b	0.93a	1.74a	3.50b	4.70b	2.46a
Spermidine	1.0	0.45a	0.38a	09.38a	0.68c	0.17a	0.29b	0.34b	0.31b	0.27a	0.28a	0.90b	0.64c
Spermine	5.1	1.16a	0.91b	0.43c	2.17d	0.21d	0.54b	0.40c	0.65d	0.34a	0.52b	0.55b	0.21a
Total	21.3	34.78a	74.41b	66.12b	36.96a	49.01a	108.13b	142.06c	59.81a	51.48a	122.67b	132.15b	51.67a

[a] C: Control batch.
[b] PE: Control batch added with 600 units/kg of Pronase E.
[c] PE + P: PE batch added with *Penicillium aurantiogriseum* extract (100.87 mg protein/kg, initial mixture); P: Control batch added with *Penicillium aurantiogriseum* extract (100.87 mg protein/kg, initial mixture).
[d] Control batch added with *Penicillium aurantiogriseum* extract (100.87 mg protein/kg, initial mixture).
[e] a, b, c: Values in a row with different letters are significantly different ($p < 0.05$).

that in the batches to which the protease was added there was more decarboxylation of the amino acids released. Pronase E is obtained from *Streptomyces griseus*. It is composed of several proteinases and aminopeptidases and a carboxipeptidase (Narahashi, Shibuya & Yanagita, 1968). Since Pronase E lacks decarboxylase activity, the increase in the amine content observed (even on fermentation stage, day 5) in batches containing this enzyme extract was probably due to an increased metabolic activity of bacteria, as a consequence of the higher availability of free amino acids released by the action of Pronase E. These results, however, disagree with those reported by Hagen, Berdagué, Hock, Næs and Blom (1996), who, after adding a proteinase (NCDO 151) of *Lactobacillus paracasei* subsp. *paracasei* to dry fermented sausages detected a lower amine content than in control fermented sausages. These authors attributed this lower content to the low pH reached in fermented sausages supplemented with the proteinase. With regards the fungal extract, this does not appear to produce significant increases in total amine content the corresponding batches (batch P vs C and batch PE + P vs PE). The amines which underwent the most pronounced increase during ripening period were putrescine, histamine + cadaverine and tyramine. The predominance of these amines agrees with results obtained by other authors in different fermented sausages (Dierick et al., 1974; Hagen et al., 1996).

Table 4 shows the results of the organic acid analysis. Total initial levels of these compounds were close to 1000 mg/100 g DM and rose during the ripening process to values of 4000–5000 mg/100 g DM In all the batches there was a sharp rise of total organic acids during the fermentation stage. The following acids were detected: citric, pyruvic, succinic, lactic, uric, formic, acetic, propionic and *n*-butyric. Lactic was the most abundant at the beginning of the ripening process, obviously coming from post-mortem anaerobic glycolysis. Pyruvic, lactic, formic + uric, acetic, propionic and *n*-butyric acids increased markedly during the fermentation stage. Citric acid increased later, after fermentation whereas succinic acid disappeared during the first few days of ripening. During the whole process, the most abundant acid, in all batches was lactic acid, reaching its highest values when the maximum growth of lactic acid bacteria was achieved, after the fermentation stage. The maximum levels of the other acids were reached around the middle of the ripening process after which they stabilised or suffered a gradual decline to the end of the ripening period. An exception to this trend was observed with propionic acid which reached maximum values at the end of ripening and even became the most abundant acid in batch P. Levels of this acid rose in all the batches although its production was clearly influenced by the presence of the fungal extract (Table 4), being the highest values found in batches PE + P and P. Propionic acid can be generated by the breakdown of sugars, lipids and proteins (Mateo & Zumalacárregui, 1996). However, given that the fungal extract did not have any lipolytic activity and that the greatest increases were observed at the end of ripening, the rise in propionic acid was probably due to the action of the extract on the amino acids.

In a study on Spanish fermented sausages, Mateo, Dominguez, Aguirrezábal and Zumalacárregui (1996a) detected similar levels of acetic and lactic acid to those recorded in control batches in this work. The apparent effect of Pronase E on the production of lactic acid during the fermentation stage (first 5 days) was noteworthy since this acid rose by approximately 30% in batches manufactured with this substance compared to the controls. This suggests that this enzyme could

Table 4
Changes in organic acids (mg/100 g D.M.) during ripening of dry fermented sausages

	Day 0	Day 5				Day 15				Day 26			
Organic acid	C[a]	C[a]	PE[b]	PE + P[c]	P[d]	C[a]	PE[b]	PE + P[c]	P[d]	C[a]	PE[b]	PE + P[c]	P[d]
Orotic	0.00	0.00a[e]	0.01b	0.01b	0.06c	0.00a	0.00a	0.00a	0.05b	0.00a	0.00a	0.05b	0.01c
Citric	12.87	10.78a	14.21b	10.74a	10.58a	25.69a	21.89b	19.84b	23.58a	25.99a	20.14b	10.87c	18.96b
Piruvic	0.89	2.87a	3.28b	3.07c	3.83d	2.04a	2.14a	1.24b	0.98b	2.12a	2.22a	2.39a	1.23b
Succinic	132.7	0.01a	4.13b	5.87c	0.10d	0.10a	2.2b	2.3b	0.11a	0.12a	1.5b	3.45c	3.00c
Lactic	852.14	1987.00a	2945.01b	2563.14c	1857.41d	2987.26a	2997.23a	2500.41b	2846.32c	2687.15a	2745.98a	2100.16b	2301.78c
Formic + Uric	3.74	30.27a	47.21b	9.63c	19.36d	63.35a	71.56b	80.22c	74.12b	55.06a	52.39a	58.27a	96.74b
Acetic	10.23	47.27a	80.27b	21.36c	24.77c	85.7a	80.27b	65.78c	78.96b	69.99a	73.24b	60.74c	99.87d
Propionic	21.06	189.96a	342.98b	512.44c	345.99b	998.15a	1109.20b	1823.96c	1798.33c	1456.21a	1189.37b	1900.15c	2433.07d
N-butyric	0.12	24.12a	29.87b	30.11b	41.07a	26.37a	32.78b	41.27c	45.71c	31.89a	30.88a	35.89a	58.93b
Total	1033.75	2292.28a	3466.97b	3156.37c	2303.17a	4188.66a	4317.27b	4535.02c	4868.16d	4328.53a	4115.72b	4171.97b	5013.59c

[a] C: Control batch.
[b] PE: Control batch added with 600 units/kg of Pronase E.
[c] PE + P: PE batch added with *Penicillium aurantiogriseum* extract (100.87 mg protein/kg, initial mixture).
[d] P: Control batch added with *Penicillium aurantiogriseum* extract (100.87 mg protein/kg, initial mixture).
[e] a, b, c: Values in a row with different letters are significantly different ($p < 0.05$).

increase microbial fermentation by increasing the amount of free amino acids available for the microbial growth. However, these differences disappear during the rest of the ripening period and there was even reductions in lactic acid at the end of this period in batches manufactured with fungal extract.

A total of 69 compounds were identified in the analysis of the volatile compounds (data not shown). Of these, 17 were hydrocarbons, 14 aldehydes, 13 terpenes, 10 alcohols, 8 ketones, 3 sulphur compounds, 2 esters and 2 acids. Edwards, Dainty and Ordóñez (1991), Edwards, Ordóñez, Dainty, Hierro and Hoz (1998) and Stahnke (1995) detected similar compounds in a Spanish fermented sausage (salchichon). In general, the batches supplemented with Pronase E had a higher concentration of volatile compounds (data not shown) and the highest values were recorded in batch PE + P.

Differences in the levels of some volatile compounds were observed over the ripening period (Table 5). Some of the compounds which presented the most pronounced differences between batches, such as 2- and 3-methylbutanal, 2- and 3-methylpropanal, are derived from the breakdown of amino acids, which in turn are broken down to produce their corresponding alcohols, 2- and 3- methyl-1-butanol (Montel, Masson & Talon, 1998). These compounds have been detected in a number of fermented sausages (Berger, Macku, German & Shibamoto, 1990; García, et al. 1991; Berdagué, Monteil, Montel & Talon, 1993; Blom et al., 1996; Mateo, Domínguez, Aguirrezábal & Zumalacárregui, 1996b) and it has been reported that 2 and 3-methylbutanal are no doubt important for the sausage aroma (Stahnke, 1994). There was a marked increase in branched chain aldehydes in the batches manufactured with Pronase E and an even greater rise in the batches which contained both the protease and the fungal extract. Also, in batches manufactured with fungal extract alone the concentration of branched aldehydes was greater than in the control. This suggests possible activity of the fungal extract on the amino acid precursors of these compounds

Table 5
Volatile compounds (TIC$\times 10^8$) that showed differences ($p < 0.05$) between batches after 26 days of ripening

	Batch			
Compound	C[a]	Pe[b]	PE + P[c]	P[d]
Acetaldehyde	+[e]	3+	3+	2+
2-Methylbutanal	+	2+	4+	2+
3-Methylbutanal	3+	5+	5+	4+
2-Methylbutenal	n.d.[f]	+	+	n.d.
2-Methylpropanal	2+	2+	3+	3+
3-Methylpropanal	+	+	3+	2+
Hexanal	5+	2+	3+	+
Benzaldehyde	+	2+	3+	+
2-Propanone	4+	5+	5+	5+
2,3-Butanedione (diacetyl)	2+	5+	5+	5+
Methylpentanone	n.d.	+	+	2+
2-Heptanone	2+	+	n.d.	+
2-Methyl-1-butanol	+	+	4+	2+
3-Methyl-1-butanol	3+	4+	4+	3+
Hexanol	n.d.	n.d.	+	+
Ethyl acetate	+	2+	2+	+
2-Methylpentanoic acid	+	2+	3+	+
3-Methylpentanoic acid	+	+	2+	+
Carbon disulphide	2+	2+	4+	3+
Dimethyl disulphide	+	+	2+	+

[a] C: Control batch.
[b] PE: Control batch added with 600 units/kg of Pronase E.
[c] PE + P: PE batch added with *Penicillium aurantiogriseum* extract (100.87 mg protein/kg, initial mixture).
[d] P: Control batch added with *Penicillium aurantiogriseum* extract (100.87 mg protein/kg, initial mixture).
[e] +: $< 1\times10^8$ Total ion current; 2+: 1×10^8–2×10^8 TIC; 3+: 2×10^8–3×10^8 TIC; 4+: 3×10^8–4×10^8 TIC; 5+: $> 4\times10^8$ TIC.
[f] n.d.: Not detected.

(Leu, Ileu and Val). These effects correspond to the marked decline of Leu and Val observed in the PE + P batch (Table 2). Blom et al. (1996) and Hagen et al. (1996) also observed an increase in the concentration of these aldehydes when a proteinase (NCDO 151) of *Lactobacillus paracasei* subsp. *paracasei* was added to fermented sausages.

Another volatile compound which differed among batches was diacetyl. This is produced in the metabolism of carbohydrate and increased significantly in the fermented sausages to which Pronase E and/or fungal extract had been added, probably due to an increase in the metabolic activity of the starter cultures due to higher avaibility of the amino acids, as discussed earlier. These results, however, contrast with the observations of Blom et al. (1996) and Hagen et al. (1996) who recorded a decline in the metabolic activity of the starter cultures after addition of a proteinase (NCDO 151) of *Lactobacillus paracasei* subsp. *paracasei* and also a reduction in this compound. A similar pattern was observed with acetaldehyde although this compound does not contribute to any major extent to the aroma of dry fermented sausages (Langner, Heckel & Malek, 1970; Halvarson, 1973).

Table 6 shows the results of the instrumental textural analysis. Two patterns with significant differences were observed in the batches (C and P vs PE and PE + P), which affected most parameters, i.e. hardness, cohesiveness, gumminess, chewiness and cutting force. These differences were not due to the fungal extract but they were attributable to the fragmentation of proteins by Pronase E, which produces a higher solubility of proteins leading to greater softness. There was a significant decrease in all the parameters, except for adhesiveness and springiness in the batches manufactured with Pronase E compared to the other batches. Also, in batch PE + P there was a significant reduction in cohesiveness ($p < 0.05$) compared to the others.

There were significant differences ($p < 0.05$) between all the batches in the triangular test for the sensory analysis (data not shown). However, only the minimum number of tasters required for the test to achieve statistical significance were able to differentiate between batches C and P.

Table 7 shows the results of the descriptive sensory analysis. There was no significant difference between the colour of the fermented sausages in the different batches. However, the odour and the flavour of sausages in batch PE + P was significantly higher ($p < 0.05$) than those of the other three batches. With regards to texture, the two batches to which Pronase E had been added (PE and PE + P) had significantly better ($p < 0.05$) texture than batches manufactured without the enzyme. The batch with the highest score in the four attributes was PE + P, followed by batches PE and P, which agrees

Table 7
Sensory analysis (mean ± standard deviation) of dry fermented sausages after 26 days of ripening

	Batch			
Characteristic	C[a]	PE[b]	PE + P[c]	P[d]
Colour	7.7 ± 0.8a[e]	7.7 ± 1.2a	7.8 ± 1.0a	7.7 ± 1.3a
Odour	6.6 ± 0.7a	7.3 ± 0.8b	8.2 ± 0.9c	7.5 ± 0.7b
Texture	6.6 ± 0.8a	8.2 ± 1.3b	8.1 ± 1.1b	7 ± 1.1a
Flavour	6.8 ± 1.0a	7.5 ± 0.9b	8.8 ± 0.5c	7.6 ± 0.6b
Overall quality[f]	6.8 ± 0.7a	7.7 ± 0.5b	8.4 ± 0.6c	7.4 ± 0.7b

[a] C: Control batch.
[b] PE: Control batch added with 600 units/kg of Pronase E.
[c] PE + P: PE batch added with *Penicillium aurantiogriseum* extract (100.87 mg protein/kg, initial mixture).
[d] P: Control batch added with *Penicillium aurantiogriseum* extract (100.87 mg protein/kg, initial mixture).
[e] a, b, c, d: Values in a row with different letters are significantly different ($p < 0.05$).
[f] Overall quality = (colour × 0.1) + (texture × 0.25) + (odour × 0.15) + (flavour × 0.5).

Table 6
Texture analysis (mean ± standard deviation) of dry fermented sausages after 26 days of ripening

	Batch			
Parameter	C[a]	PE[b]	PE + P[c]	P[d]
Hardness (N)	269.2 ± 9.5a[e]	167.3 ± 12.8b	166.8 ± 6.8b	273.2 ± 4.3a
Adhesiviness (m^2)	−0.85 ± 0.3a	−1.28 ± 0.5a	−1.04 ± 0.3a	−0.75 ± 0.1a
Springiness (m)	0.00686 ± 0.001a	0.00646 ± 0.001a	0.00587 ± 0.004a	0.00660 ± 0.003a
Cohesiviness	0.379 ± 0.009a,b	0.346 ± 0.02a,c	0.320 ± 0.02c	0.385 ± 0.007a
Gumminess (N)	102.1 ± 5.2a	57.8 ± 5.3b	53.4 ± 5.4b	105.1 ± 3a
Chewiness (J)	0.703 ± 0,011a	0,373 ± 0,05b	0,313 ± 0,033b	0,694 ± 0,037a
Cutting force (N)	156.4 ± 3.1a	110.2 ± 12.2b	102.1 ± 2.5b	158.51 ± 2.8a
Cutting work (m^2)	0.00148 ± 0.0001a	0.00929 ± 0.0001a	0.00813 ± 0.0003a	0.001397 ± 0.0002a

[a] C: Control batch.
[b] PE: Control batch added with 600 units/kg, of Pronase E.
[c] PE + P: PE batch added with *Penicillium aurantiogriseum* extract (100.87 mg protein/kg, initial mixture).
[d] P: Control batch added with *Penicillium aurantiogriseum* extract (100.87 mg protein/kg, initial mixture).
[e] a, b: Values in a row with different letters are significantly different ($p < 0.05$).

with the textural analysis. The overall acceptability of the PE + P batch was significantly higher ($p < 0.05$) than that of PE and P and also much higher than that of the control batch. These results are more satisfactory than previous reported by Bruna et al. (1999) because a higher acceptability was obtained. This probably indicates higher degradation of free amino acids by *Penicillium aurantiogriseum* extract, promoting a higher release of compounds contributing to dry sausage flavour.

4. Conclusions

As expected, addition of Pronase E generates important changes in the composition and sensory properties of fermented sausages. The addition of the fungal extract alone produced slight changes in the composition of the fermented sausages, reflecting an improvement of sensory characteristics. In the absence of changes in the free fatty acids, this improvement was attributed to the effect of the *Penicillium aurantiogriseum* extract on the free amino acids. These compounds could be transformed into ammonia or keto-acids by the action of the enzyme in the extract or into other volatile compounds, namely, 2- and 3-methylbutanal, 2- and 3-methylpropanal and 2- and 3-methyl-1-butanol.

In conclusion, the combined action of a protease (in this case Pronase E) and a fungal extract (*Penicillium aurantiogriseum*) improves the sensory characteristics of dry fermented sausages.

Acknowledgements

This work was funded by the Comisión Interministerial de Ciencia y Tecnología (CICYT) with the project ALI 96-0928. J.M.B. was awarded a predoctoral scholarship of Universidad Complutense de Madrid.

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